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Synergistic Impact of Copper Supplementation and Electricity on Regenerative Potential in Earthworms

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***Abstract*—Earthworms are the most commonly known organisms to possess extraordinary capability of regeneration by regenerating their missing body parts post amputation at certain segments. It conscripts a program of wound healing, dedifferentiation, blastema formation and cellular differentiation and are good models for regenerative processes. Trace elements are important for regeneration in addition to other biotic and abiotic factors. Copper is an essential trace element in earthworms, playing roles as a nutrient and toxicological factor. Copper is an essential cofactor for a number of enzymes that participate in enzymatic reactions, which influences the regulation of oxidative stress, collagen cross-linking, wound repair and angiogenesis. Despite its essentiality, the potential toxicity of gaining high copper levels raises important questions about dosages and cellular responses. The present review has focused on the synergistic effect of copper supplementation in form of copper II sulfate pentahydrate and electrical stimulation for regeneration in earthworms by comprising current state of knowledge and evidence of experimentation. Conclusions and future research directions. The review outlines potential areas where copper-mediated processes might apply in regenerative biology and underscores the need for comparative research to dissect these mechanisms, including the application of standardized protocols, long-term ecological consequences of exposure to elevated copper levels (vs. conditions applied in regenerative studies), molecular-level knowledge required for interpreting observed effects of copper on regeneration biology, and insights into evolutionary-conserved versus specific spatiotemporal signalling networks that underpin these effects. Regeneration is an important biological process in which a precisely controlled series of cellular events allow the repair or complete regrowth of lost tissues. as proliferation, migration, and extracellular matrix remodeling. Recent studies indicate that trace metals and bioelectric cues play complementary roles in regulating these processes. The present study explores the synergistic action of copper supplementation and controlled electrical stimulation upon regeneration in two disparate biological systems: earthworms (a classic in vivo model for regenerative biology). In the earthworm model, copper sulphate was added at subtoxic concentrations following transverse amputation, and specimens were subjected to low intensity DC stimulation. Regenerative advancement was assessed through morphological measurements, histological screening and quantification of neoblast proliferation. Outcomes revealed that copper supplementation alone enhanced early blastema formation, likely due to copper's function as a cofactor within lysyl oxidase and antioxidant enzymes. Electrical stimulation independently accelerated epithelial closure and neoblast migration. Notably, combined treatment produced a far quicker and more directed regenerative response, suggesting that copper- enhanced enzymatic activity and bioelectric signalling cooperate in the improvement of tissue patterning. .Low-dose copper enhanced collagen synthesis and (Reactive Oxygen Species) ROS-mediated signaling associated with matrix remodeling, while electrical stimulation promoted directional migration, further enhancing cytoskeletal organization Copper supplementation and pulsed electrical stimulation act synergically, providing the highest proliferation index, increasing the expression of markers related to wound healing (COL1A1, TGF- β1), and mitochondrial activity in the absence of cytotoxicity induction.**

***Index Terms*—Copper (II) sulfate pentahydrate, Earthworms Regeneration, Toxicity, Wound-repair**

I. INTRODUCTION

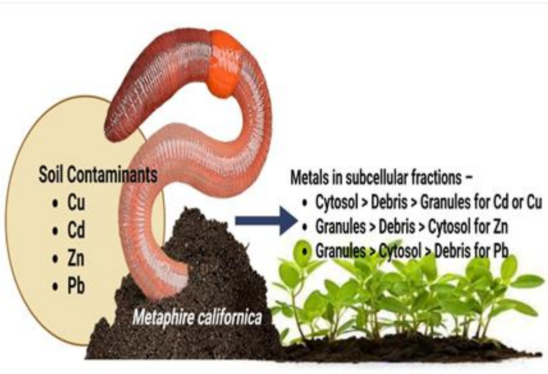

Fig: 1.1: Role of the soil contaminants

Earthworms are eucoelomate invertebrates, multicellular that live in terrestrial environment. They are a major part of soil macrofauna. These act as major bioindicators, helping study the physio chemical aspects of their habitat. Variation in species richness, abundance, and distributional patterns demonstrates the impact of variations in soil and climate over different geographic zones. They contribute majorly to pedogenesis and soil profile because of their horizontal and vertical arrangement of segments. They enrich soil with organic matter to improve soil fertility by shallow ploughing. They contribute to soil aeration and water infiltrations. They are important source of food for organisms like birds or moles. Notably, they are also easy to collect and breed (Kale & Karmegam, 2010). An earthworms' body is divided as 120 segments with three regions namely, Head, Clitellum and intestine. The pre-clitellum segments are present in head region that carry out primary functions. A pair of prostate glands are auxiliary reproductive glands, present next to clitellum (Selvan Christyraj et al., 2025). Copper (Cu) is a trace element present in earthworms. In high concentration, it becomes harmful. At recommended dosage, copper serves as a cofactor for key enzymes including superoxide dismutase, lysyl oxidase, and cytochrome c oxidase and, thus, plays an important role in antioxidant defense, collagen cross-linking, energy production, and cell growth. Such roles are beneficial in wound healing and tissue regeneration. This equilibrium of balance is vital in the study of earthworm biology to gain insight into both the ecosystems within soil and the environmental factors affecting those ecosystems. For countless years, scientists have been fascinated by the idea of tissue regeneration at will. Humans may regenerate skin after being cut, but our regenerative ability is significantly less than other larger species like salamanders and earthworms. Scientists will find new methods for treating and regenerating human tissue through their investigation of the regenerative processes in other species. The work done by researchers on developing new methods for wound healing and regenerative medicine has revealed that the process of regeneration is not simply a function of one particular factor, but instead consists of several different factors which include biochemical signals, bioelectrical signals and cellular signals. For example, copper (Cu) is an important trace mineral for all living organisms because of its role in many developmental processes, including wound healing. Wound Healing and Bioelectricity: Copper's Role Copper is an essential metal mineral that is involved in the wound process. The use of copper aids in the action of copper-dependent enzymes such as lysyl oxidase which bulks up collagen and elastin as well as providing increased strength to newly formed tissues (osteoclasts, chondrocytes) by enhancing cellular antioxidant systems (superoxide dismutase SOD). Copper also plays a key role in morphological development by playing a role in the development of healthy tissue through proper morphogenesis of tissues after injury. While the biological role of copper has been established for years, there has been little research on the ability of copper to enhance the regeneration process regardless of whether it is used alone or in combination with other biological stimuli. Recently, another powerful regulator of healing has been researched; bioelectricity. Bioelectric fields are produced naturally following an injury, with these small electric fields acting to direct cells toward an injury site, to direct the proper alignment of cells to aid in proper repair, and activate multiple cellular (pathways) within the wound repair apparatus. While electrical stimulation appears to serve as an alternative to the body's own signals that are normally utilized to regenerate or repair tissues, the manner in which electrical stimulation interacts with other biochemical signals (e.g., copper), which also aid repair through development of connective tissue, still requires further investigation. The goal of this project is to better understand how copper and electrical stimulation work together to stimulate tissue repair by using both an in vivo model (e.g., earthworm). The hypothesis is that copper assists in the biochemical signaling process (e.g., collagen production) involved in tissue repair by providing additional strength or support to the signaling process, while electrical stimulation facilitates or directs cellular response to these biochemical signals (e.g. copper). If there is a synergistic effect through the use of these two signals/mediators, then by combining the two signals/mediators there is the potential to dramatically increase tissue regeneration success. Ultimately, this research will hopefully contribute to the development of new healing methods through the use of copper-based regenerative medicine applications (e.g., currently being investigated) or through the development of bioelectrically active wound dressings.

II. WORM REGENERATION: AN OVERVIEW

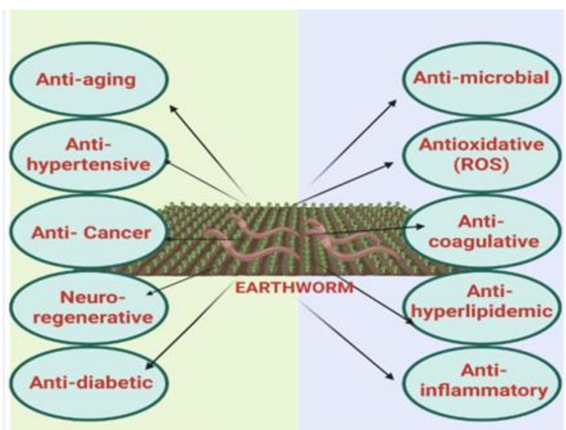

Fig:2.1: earthworm uses

Regeneration is a significant ability of an organism to heal and replace its missing body parts. Annelids, nemertean worms, planarians and chordates are few examples with extensive regenerative abilities. Earthworms play a major role in soil formation, maintenance and structure and turnover of dead organic matter. These factors influence their survival. Annelids have been used commonly to study regeneration mechanisms (Myohara et al., 1999). Oligochaetes and polychaetes, can regenerate their lost body segments. In annelids, the lumbricid earthworm, Eisenia fetida, play a vital role in conversion of organic wastes, used commonly for research regeneration, as it is easy handle in the laboratory. It involves wound healing, dedifferentiation, blastema formation and cellular differentiation (Bely & Wray, 2001). They have a true coelom, metamere, a vascular system, brain ganglia, longitudinal cords and sense organs. Earthworms are often cut into parts by predators; hence it is necessary to know the regeneration abilities for their survival (Xiao et al., 2011). Clitellum is the main part responsible for regeneration. The clitellum's growth is influenced by feed provided. The growth starts from 27 – 33 days after hatching (Kabi et al., 2020). Stem cells can be observed within the first week especially in juvenile cells implying that juvenile worms can regenerate efficiently than mature adult worms (Rajagopalan et al., 2022). The regeneration capacity is affected by environmental factors such as temperature and nutrition. Earthworms cultured at 25 °C regenerated faster than those kept at 30 °C and 20 °C (Liebmann, 1942). It is also stated that sexual activity can also influence rates of regeneration, and this influence decreases progressively, the farther posterior from the sexual organs is the point of amputation. Although regeneration is so important in earthworm ecology, little research on earthworm regeneration has been reported since the 1980s (Xiao et al., 2011). Exposure of earthworms to unfavorable conditions results in changes in their external morphology. They get coiled up in water, where clitellum becomes more noticeable as it turns red. (Zirbes et al., 2012) Likewise when they are exposed to 10% ethanol, they undergo stress and secrete coelomic fluid. Also elevated levels of ethanol might lead to the death of the worm. A clear swelling of clitellum can be observed upon exposure of the worm to toxic or pollutant substances (Subbiahanadar Chelladurai et al., 2020). Over swelling or bulging in clitellum due to stress causes inflammation and internal bleeding which causes death of the worm. In arid soil, worms face temporary modifications in their functions and reproductive capabilities of earthworms. They can regain their functions once after the conditions become normal (Rajagopalan et al., 2022).

III. ROLE OF TRACE ELEMENTS IN REGENERATION

Trace elements have an essential role in the chemistry of the cell and in the regenerative process of the cell. Trace elements in the body serve a variety of purposes by providing cofactor support to enzymes, providing structural support, and assisting with the maintenance of oxidation balance (Rae et al., 1999). Copper is found in two forms: Cu1+ (cuprous) and Cu2+ (cupric), which serve different functions. Cuprous copper transports electrons within the mitochondria and is linked to the form of collagen and healing of wounds, while cupric copper functions in the same way but is also a cofactor for many enzymes (Rae et al, 1999). Approximately 300 enzymes and 1000 transcription factors utilize zinc as a functional cofactor and are needed to synthesize DNA and metabolize protein, as well as in cellular production and dedifferentiation during the processes of both healing and regeneration (Prasad, 2014). In addition to facilitating oxygen transport throughout the body, iron provides the energy used by mitochondrial respiration for the production of ATP, and it is vital in order for the enzymes to be active. Iron is needed in both the process of degeneration and regeneration of nervous tissue (Todorich et al., 2009). As co-factors and because of its presence in the antioxidant enzyme system manganese (e.g., manganese-superoxide dismutase) plays a critical role in protecting tissue against oxidative damage and from degeneration. Iron is the most abundant element followed by zinc and copper and it has been proved by trace metal content in neural tissues. This highly contributes to regenerative capacity and physiological significance. Deficiencies in Copper, Iron, Zinc or Manganese cause proteomic and metabolic alterations. Thus, these are important for cellular growth and stress tolerance (Naumann et al., 2007). Peripheral nerve injury, iron accumulation during degeneration and subsequent reduction during regeneration prove trace metal involvement in tissue repair and healing (Huang et al., 2025). Hence these prove that metals are essential in regeneration abilities of earthworm and their molecular mechanisms like nutrition uptake, utilization and toxicity.

IV. COPPER: A DOUBLE-EDGED SWORD

Copper is known to be an essential micronutrient for the growth and development of earthworms and when it is present at certain concentrations an earthworm may either be a valuable nutrient or a toxicant. Copper also serves as an important cofactor in the functioning of a variety of different types of enzymes related to oxidative stress, collagen cross-linking, cell proliferation, wound healing, and angiogenesis, in addition to all processes associated with regeneration following injury (Peña et al. 1999). Earthworms improve the soil's overall health, but they are not only known for aerating soil and breaking down organic materials through their burrows (Bohlen 1996). At high concentrations copper will produce reactive species (R.S.), which have been shown to cause severe damage to living organisms. When administered in controlled doses at very low concentrations, copper can enhance the process of regeneration by providing strength to the extracellular matrix (ECM), stimulating the formation of new blood vessels, reducing their oxidative stress, and enabling the rapid migration and growth of cells. These benefits of copper occur in simple organisms such as earthworms. Copper's positive influence or negative effect will be enhanced by the electrical field. The other downside of copper is that it reduces the reactive oxygen species (ROS)-scavenging or antioxidant protection within cells through its depletion of glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in earthworms exposed to oxidative injury caused by ROS (Bohlen,1996). Additionally, copper supplementation enhances cellular antioxidant defense by stimulating antioxidant enzyme production, thereby protecting cells from free radical damage (Gaetke & Chow, 2003) exhibiting a “double-edged sword” effect in earthworms. Discovering safe levels of copper supplementation is necessary for assessing risks of copper accumulation in soils as earthworms (Nahmani et al., 2007).

V. EXPERIMENTAL STUDIES ON COPPER SUPPLEMENTATION AND REGENERATION

Experimental studies have shown that the effects of copper supplementation on earthworms are highly based on exposure route, dose, and duration. Copper salts such as copper sulfate or copper oxychloride are used to simulate agricultural contamination. Enzymatic disruptions and oxidative stress occur at even lower levels. Lethal concentration (LC_{50}) is around 113 mg Cu/kg (dry soil), reproduction EC_{50} near 95 mg/kg, and growth EC_{50} about 144 mg/kg (dry soil). Most laboratory experiments that look at copper supplementation depend on widely available copper salts, including copper sulfate ($CuSO_4$) or copper oxychloride, found in agricultural soils due to pesticide and fungicide applications. For example, several studies demonstrate a slight stimulation of growth, enzyme activity, or metabolic activity in earthworms when they are exposed to approximately 80 mg Cu/kg of dry soil. Toxicity studies commonly report that the LC_{50} (the concentration lethal to half the Studies indicate that the typical concentration at which Earthworms can survive with copper in soil is around 113 mg Cu/kg. However, prior to reaching lethal levels of copper, many functions that are vital to Earthworms including reproductive and developmental processes, experience declines, which inhibits Earthworm reproductive and growth processes. These declines in reproductive and developmental processes create a danger to the long-term viability of Earthworm populations and the stability of the soil ecosystem in contaminated areas. In natural soils, copper may bind to Humic Substances, Clay or Organic Residue, reducing the immediate toxic effects of copper. Copper's effect on the expression of genes associated with mitochondria, and subsequently, its effect on pathways used to create energy, leads to the requirement of an organism using most of its energy for detoxifying from or defending against copper instead of to grow or repair itself.

VI. MECHANISMS UNDERLYING COPPER'S IMPACT

When earthworms were exposed to nonlethal concentrations of copper in soil, they exhibited multiple signs of damage such as increased amounts of certain stress-response genes (including metallothionein, heat shock proteins, superoxide dismutase, and catalase) and increased amounts of various antimicrobial peptide genes (including FET and LYS). This suggests that earthworms exposed to these nonlethal copper concentrations activated their immune and detoxifying mechanisms (Mincarelli, [Date of publication was not provided]). When earthworms were exposed to higher concentrations of copper, they responded by disrupting their basic metabolic processes. Transcriptome and metabolome analyses (based on RNA and metabolite profiling) indicate that copper exposure alters the normal amino acid, lipid, carbohydrate, and energy events involved with the utilization of nutrients and the production of antioxidants in Eisenia fetida. In addition, copper exposure reduces the diversity of the gut microbiome and affects their ability to absorb nutrients and produce antioxidants (Zhang et al., [Date of publication was not provided]). Copper's impact on living systems arises from a mix of essential biological roles These illustrate how copper influences molecular, cellular, and immunological systems in earthworms, affecting viability, regeneration, and environmental health.

VII. ENVIRONMENTAL AND ECOLOGICAL IMPLICATIONS

Both as a useful substance and as a harmful pollutant, copper has a large impact on the health of earthworm populations and, by extension, the health of soils through its ability to support fungicidal protection when applied at the correct concentration levels in agricultural soils. Accumulation of copper after repeated applications increases soil contamination, which has negative impacts on root development, imparts harmful effects to fungi and bacterial communities, and negatively impacts earthworm populations. Earthworms are among the most sensitive organisms to copper contamination; as a result, they are often used as bioindicators of soil contamination. The most common species used for agricultural assessments is Eisenia fetida, which has been shown to exhibit decreased responses when total copper exceeds 240 mg/kg. High levels of copper negatively affect reproduction, survival, growth, and behavior, resulting in reduced cocoon production, decreased enzyme production, altered burrowing behavior, and decreased biomass and community diversity. From an environmental perspective, the interaction between copper as a beneficial micronutrient and as a harmful pollutant is complex, depending on its accumulation levels in soil and the responses of organisms such as earthworms. Earthworms are critical components of ecological engineering processes that maintain balanced soil ecology. Copper pollution has been found to affect earthworm behavior, leading to declines in spawning numbers, size, cocoon production, and increased mortality over time. These changes reflect a decline in the health of earthworm populations and indicate the level of stress imposed on the soil ecosystem. The ecological implications of copper pollution in predator–prey interactions involving earthworms are not well known. Birds, reptiles, amphibians, and small mammals depend heavily on earthworms as a food source; therefore, copper accumulated in earthworm tissues can be transferred to predators, resulting in biomagnification through the food web. While earthworms can regulate metal accumulation within their bodies to some extent, they cannot prevent metal transfer to their predators, highlighting the need for further ecological investigation. Some earthworm species exhibit genetic adaptation to chronic copper exposure, showing faster growth, earlier maturity, and higher reproduction in copper-polluted environments, sometimes at the expense of increased mortality (Fisker et al., 2011). These findings are significant for vermiculture and soil health, as earthworms play a vital role in nutrient cycling, soil structuring, and organic matter decomposition (Usmani & Kumar, 2015).

VIII. CHALLENGES AND RESEARCH GAPS

Copper is an essential element in earthworm biology; however, most research on the impact of copper has been in vitro based or toxicity focused, with limited emphasis on the molecular and cellular mechanisms through which copper affects regeneration in earthworms. Moreover, most studies conducted so far lack standardized experimental protocols or exposure conditions, resulting in considerable variability due to differences in soil type, pH, organic matter content, and copper concentration used for exposure (Lukkari et al., 2004). Another area where research continues to be lacking is the detailed molecular and genetic role of copper. The only available information regarding copper's effects on gene regulation, signal transduction, and epigenetic modifications during tissue repair and regeneration is its role as a cofactor for the enzyme's superoxide dismutase and lysyl oxidase (Spurgeon et al., 2004). The application of modern molecular biology techniques, such as transcriptomics, proteomics, and CRISPR-based knockdown studies, has the potential to provide a deeper understanding of the mechanisms by which copper influences earthworm physiology and to clarify its beneficial and toxic effects. Despite years of research on copper ion exposure in earthworms, there remains a significant knowledge gap and a lack of integration between ecological and biomedical perspectives. Copper's dual role as an essential micronutrient and a toxicant creates uncertainty in interpreting its effects across biological and environmental systems. Current understanding of copper remains incomplete, particularly regarding its specific impact on regeneration at the molecular and cellular levels, the mechanisms underlying species-specific tolerance to elevated copper levels, and the broader ecological consequences of increased copper accumulation in soils. One of the major gaps in copper research is the lack of direct mechanistic studies examining the effects of copper supplementation on the regenerative process in earthworms. Much of the existing research documents copper's toxic effects, such as oxidative stress and enzyme repression, rather than its role in regeneration. While some studies have explored associations between copper exposure and regeneration, there is a substantial lack of in-depth research addressing how copper influences the sequential stages of biological regeneration, including wound healing, blastema formation, stem cell mobilization and activation, cell differentiation, and tissue remodeling. Most existing information is derived from toxicological or observational studies rather than well-designed experiments that directly examine regenerative processes at the tissue level. Consequently, conclusions regarding copper's effects on regeneration are often inferred indirectly from stress-related biological markers rather than from direct observation of regenerative events as they occur. This limitation is further compounded by the absence of universally accepted standardized protocols for assessing the effects of copper exposure on regenerative tissues over time.

IX. FUTURE PERSPECTIVES

Future research on copper in earthworms should prioritize sustainable and controlled supplementation strategies that balance copper's essential biological role with its potential toxicity. Chelated and nanoparticle-based copper formulations are being explored to improve bioavailability while limiting freeion toxicity. However, the long-term ecological consequences of nanoparticle copper remain insufficiently understood. Environmental engineering approaches, such as earthworm-assisted vermicomposting systems, offer promising strategies for copper management in waste treatment and soil remediation. Advances in analytical and molecular technologies have improved the ability to monitor copper accumulation and to study its effects on regeneration, oxidative stress regulation, and metal homeostasis in earthworms. The application of transcriptomic and proteomic tools can aid in identifying biomarkers of copper-induced stress and help define supplementation ranges that support regeneration without adverse ecological effects. Overall, future studies should adopt multidisciplinary approaches integrating vermitechnology, nanotechnology, and molecular biology to develop ecologically sound and mechanistically informed frameworks for copper use in soil ecosystems.

X. CONCLUSION

Earthworms exhibit robust regenerative capacity through wound healing, blastema formation, and cellular differentiation (Bely & Wray, 2001; Myohara et al., 1999), a process influenced by environmental conditions and micronutrient availability. Copper plays a dual role in regeneration: at optimal levels it supports enzymatic functions related to oxidative stress regulation, collagen cross-linking, and tissue repair (Peña et al., 1999), whereas excessive copper induces oxidative damage, suppresses immune responses, and impairs regeneration (Gaetke & Chow, 2003; Nahmani et al., 2007). Effective regulation of copper exposure is therefore essential for maintaining earthworm health and soil ecosystem stability. Future research should focus on defining safe copper thresholds, integrating vermitechnology with soil remediation strategies, and applying molecular approaches to clarify the mechanisms underlying regeneration. Overall, copper's effects in earthworms highlight the importance of maintaining a precise balance between essentiality and toxicity in ecological and regenerative biology

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